Dear Editor,

For your kind consideration, I am submitting the original research manuscript entitled “**Codon optimized *alanine aminotransferase* gene infer improvement in nitrogen use efficiency in tobacco under different N regimes**” by Ahmed et al. in your journal for possible publication. This manuscript is according to scope and style of said journal and there is no conflict of interest among contributing authors. Following are the key features of this research:

1. We are reporting improvement in nitrogen use efficiency in transgenic tobacco through transformation of codon optimized alanine aminotransferase gene for the first time.
2. Plant physiological data (leaf area, stalk diameter, grain yield, plant fresh/dry weight, root dry weight), indicating the improvement in nitrogen uptake
3. Plant biochemical data (total free amino acids, total nitrogen content, crude proteins and AlaAT activity), supporting the hypothesis of improving NUE through synthetic (co-AlaAT) gene.

Thanking you in anticipation and best regards!

Moddassir Ahmed

**Codon optimized *alanine aminotransferase* gene infer improvement in nitrogen use efficiency in tobacco under different N regimes**

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**Abstract**

Improvement in nitrogen use efficiency remains a major challenge in crop plants. A barley *alanine aminotransferase* (*HvAlaAT*) gene was codon optimized to achieve optimal expression in tobacco plants. Codon optimized *alanine aminotransferase* (co-*AlaAT*) gene expressed under 2X*CaMV35S* promoter in three tobacco lines. Regenerated transgenic tobacco lines were evaluated at 0, 75, 100 and 125 Kg N/ha nitrogen fertilizer doses applied in three split applications. Molecular analysis of the putative transgenic tobacco plants were conducted through PCR and real-time quantitative PCR. Transgenic tobacco lines evaluated through biochemical and morpho-physiological means showing improvement in N uptake, total free amino acid, crude proteins, AlaAT activity, leaf area, stalk diameter, above the ground fresh mass, above ground dry matter and seed weight. All these attributes demonstrate improved nitrogen uptake and incorporating it to biomass as compared to the wild-type. From these finding, it is concluded that codon optimized *alanine aminotransferase* (co-*AlaAT*) gene could be a possible candidate for developing nitrogen use efficient crop plants, to reduce nitrate contamination in the environment and to minimize the farmer's input in term of nitrogenous fertilizer application.

**Keywords**: Biomass, crop productivity, gene synthesis, N metabolism, nutrient efficient tobacco.

1. **Introduction**

With the advancement in plant molecular biology, synthetic biological approaches are being used routinely to improve crop productivity. Since last 50 years, N fertilizers are being used quite extensively to enhance or to sustain crop yield (Mulvaney et al. 2009; McAllister et al. 2012). Although N is an essential element but usually it becomes limiting nutrient for plant growth and development due to leaching of nitrate and volatilization process (Kraiser et al. 2011). Only 30-50% of applied N is taken up by plants while the remaining is lost through ammonia volatilization, surface runoff, consumed by soil microbes and by nitrate leaching (Garnett et al. 2009). The excessive use of N fertilizer cause imbalance of other essential nutrients necessary for optimum plant growth, contribute toward greenhouse gas emission, nitrate accumulation in underground water used for drinking and cause serious threats to human health (Ahmed et al. 2017). This situation urges the strong demand for the development of nitrogen use efficient crops. During vegetative growth, plants utilize N in three steps: uptake, translocation, and assimilation. Nitrate and ammonium are the major forms of inorganic N present in agricultural soil (Howitt and Udvardi 2000). Nitrate is the main form of N consumed by many plant species. When nitrate enters the plant cell, it is reduced to nitrite by Nitrate Reductase (NR). Nitrite is transported further to plastids where it undergoes reduction process and converted to ammonia by nitrite reductase. Ammonia then enters the GOGAT (glutamine-oxoglutarate aminotransferase) cycle and transformed to glutamine and glutamate (Andrews et al. 2004). The amino group of glutamate could then be transferred to various amino acids by different aminotransferases (Lam et al. 1996). Alanine aminotransferase catalyzes the reversible conversion of alanine and 2-oxoglutarate to pyruvate and glutamate. Alanine is an important amino acid for various reasons (Good et al. 2007; Shrawat et al. 2008) e.g. it is usually excreted by N fixing bacteria and then assimilated into plant roots, suggesting its role in organic N metabolism. Additionally, under certain stresses, alanine has been found to be among the major storage amino acids (Good et al. 2007). In most of the cases, 50-75% of applied N to arable lands is utilized by microorganisms or lost through leaching (Hodge et al. 2000). So the improvement in plant nitrogen use efficiency through genetic engineering has been the focus of research since last decade. Over expression of barley alanine aminotransferase (*AlaAT*) gene in canola and rice has been demonstrated with improved nitrogen use efficiency as indicated by measured biomass and grain yield under low nitrogen supply (0.5-4.0 mM) in growth chambers, hydroponics and field trials (Good et al. 2004; Shrawat et al. 2008). A nitrogen efficient phenotype was observed in transgenic rice transformed with barley (*HvAlaAT*) gene and assessed physiologically under low, medium and high N supply (Beatty et al. 2013). Similarly, transgenic sugarcane expressing *HvAlaAT* gene were developed and assessed under low nitrogen supply, in pot experiments, which indicated improvement in nitrogen use efficiency (Snyman et al. 2015). However codon optimization of barley *alanine aminotransferase* (*AlaAT*) gene by replacing less frequently predicted codons with most favored codons to achieve optimal transcriptional efficiency and translational ability in tobacco (*Nicotiana tabacum* L.) plants (dicot) is used for the first time to be expressed under *2XCaMV35S* promoter. It was hypothesized that codon optimization of the nucleotide sequence of barley *AlaAT* gene could be an effective approach to attain optimal expression level in the targeted plant species.

1. **Materials and Methods**

**2.1 Cloning in binary vector and transformation of tobacco**

Barley *alanine aminotransferase* gene (Accession no. Z26322) was codon optimized by replacing codon predicted to be less frequently use in tobacco with more favored codons according to Codon Usage Database (http://www.kazusa.or.jp). Codon optimization was performed by keeping in view a range of factors involved in protein expression, codon adaptability, mRNA structure, various cis-elements in transcription and translation to have optimum transcriptional competence, translational efficiency and protein folding in tobacco (*Nicotiana tabacum* L.) plants. The codon optimized (co-*AlaAT*) gene along with the introduction of *Hind*III and *EcoR*I restriction sites was got synthesized commercially from M/S Eurofins Genomics, USA and delivered in general plasmid vector (*pBluescript*). The *pBluescript* plasmid was restricted with *Hind*III and *Eco*RI restriction enzymes to ligate the codon optimized *alanine aminotransferase* (co-*AlaAT*) gene (1453bp) into pJIT60 vector under *2XCaMV35S* promoter and *CaMV* terminator. Then *pJIT60* vector having the whole cassette (*2XCaMV35S*- co-*AlaAT*- *CaMV*) was digested with *KpnI* and *XhoI* hexa cutter enzymes and sub-cloned the complete cassette in a *pGreen0029* binary vector by using the same restriction sites. This construct was transformed into *Agrobacterium tumefaciens* strain ‘LBA4404’ through electroporation method. Freshly cut tobacco leaf discs were inoculated with *Agrobacterium* culture and incubated on MS0 medium (Murashige and Skoog 1962). After shoot and root development, the regenerated plants were transplanted to the soil in pots and three putative transgenic lines were selected to raise T1, T2, and T3 generations. These lines were selected to be tested further on the basis of their performance at different nitrogen regimes. The selected lines i.e. SAC1, SAC2, and SAC5 were grown in four replicates in pot experiments. Different N regimes were created by applying urea as N fertilizer at the rate of 0, 75, 100 and 125 kg N/ha and represented as N0, N75, N100 and N125 respectively. Urea fertilizer was applied in three split applications at transplantation, vegetative growth phase and flowering stage. The other fertilizers i.e. phosphorous and potassium were applied ten days after sowing at a rate of 75 kg/ha P2O5 and 50 kg/ha K2O. DNA was extracted from leaf tissues of putative transgenic tobacco plants by CTAB method (Doyle and Doyle, 1987) and tested for codon optimized alanine aminotransferase (co-*AlaAT*) gene insertion in tobacco genome by PCR. Gene-specific primers (Table 1) were used to amplify co-*AlaAT* gene with the forward (*AlaAT* AF) and reverse (*AlaAT* BR) primers in a PCR reaction of 25 µl volume containing 100 ng plant DNA, 200 µM MgCl2, 200 nM of each primer and Dream Taq Green Mix (Fermentas). The profile used for PCR reaction consisted of [Initial denaturation temperature 94°C for 4 min; 35 cycles of denaturation, (94 °C for 1 min), annealing (55°C for 1 min) and , extension (72°C for 1 min) followed by final extension at 72°C for 10 min]. For expression analysis, RNA was extracted from fully developed leaf tissues by using Trizol Reagent (Invitrogen, USA) and cDNA was synthesized by RevertAid reverse transcriptase (Fermentas, USA). Specific forward (*AlaAT* F) and reverse (*AlaAT* R) primers were used to confirm relative expression of co-*AlaAT* gene in transgenic lines through quantitative real time PCR analysis, while primer pair of *18SRNA* gene was used as reference control to compare relative expression of co-*AlaAT* gene in different transgenic lines. Real-time PCR was performed in 20 µl reaction mixture containing 50 ng cDNA, 200 µM MgCl2, 200 nM of each primer and SYBR Green qPCR kit (Fermentas, USA) using CFX-96 real-time PCR machine (Biorad, USA).

**2.2 Studies on morpho-physiological and biochemical parameters of transgenic plants**

Plant height of putative tobacco plants were measured at rapid vegetative growth stage by plant height measuring scale. Leaf area of fully developed fifth leaf was measured through CI-202 Laser Area Meter (CID Bio-Sciences, USA). Stalk diameter was recorded with a diameter measuring tape. Shoot fresh/dry weights and root dry weights were recorded just after harvesting and drying plants at 65 ºC for 72 hours in drying oven. Seed weight of each tobacco line was determined using lab scale analytical balance.

Estimation of the total free amino acids was recorded by following Hamilton and Van Slyke (1943) method. Fresh leaf tissues (1.0 g) were sliced in citrate buffer (10 mL; pH 5.0) and incubated at room temperature for 1h. This mixture was centrifuged at 15,000 rpm for 10 min at 15 ºC. The supernatant was taken carefully and used to quantify total free amino acids. One milliliter volume of the eluent was taken in a 20 mL test tube and 1 mL of ninhydrin solution was added. These tubes were kept wrapped in aluminum foil to avoid light contact and heated for 20 minutes at 100 ºC in a water bath. These tubes were then cooled down to room temperature and 5 mL of the diluent was mixed and incubated again for 15 minutes at room temperature. The optical density (OD) was measured at 570 nm on a UV-visible spectrophotometer (Hitachi-220, Japan).

The total nitrogen content of transgenic plant material was measured by following micro–Kjeldhal’s method (Bremner, 1965). The tissue samples of tobacco plants were digested with sulfuric acid (5 mL) and transferred in Kjeldhal’s tubes before placing them in Kjeldhal’s ammonia distillation unit and 5 mL of 40% sodium hydroxide (NaOH) was added to every tube. Boric acid solution (5 mL) was taken in a conical flask with few drops of mixed indicator. When the distillate reached approximately 40 mL, stopped the distillation process and let the distillate cooled down for few minutes and titrated with 0.01 N standard H2SO4 till the solution turned pink. A blank control was run during the whole procedure. The values were taken three times for a single sample to rule out the possibility of analytical error.

N content was estimated by using the following formula

N% = (V2-V1) x N x 0.014 x 100

W

Where

V2: Volume of H2SO4 used for sample during titration

V1: Volume of H2SO4 used for blank control during titration

N: Normality of the acid used for titration

N%: Percent nitrogen

After estimation of total nitrogen content, the value of crude protein was calculated by multiplying the obtained data with a conversion factor of 6.25.

Alanine aminotransferase activity was measured from freshly harvested leaf tissues of transgenic and wild-type control (as described by McAllister and Good, 2015). Harvested leaf samples equal to 5.0 g weight were ground with pestle and mortar by keeping them on ice with extraction buffer, a pinch of sand and PVPP was added. The ground samples were centrifuged for 15 min at 15.7 relative centrifugal force. The supernatant from each replicate was column purified through Amicon Filter Column and AlaAT activity was assessed in a continuous coupled reaction catalyzed by lactate dehydrogenase and AlaAT. The change of absorbance due to the formation of NAD+ from NADH was monitored at 340 nm as reported by Miyashita et al. (2007).

1. **Results**

**3.1 Selection of regenerating putative transgenic plantlets**

The putative transgenic tobacco (*Nicotiana tabacum* L.) plantlets having co-*AlaAT* gene were selected on media containing kanamycin 100 mg/L. The regeneration potential was found to be 72% while observed transformation efficiency was 49%. T1-T3 generations of transgenic tobacco lines were raised in pots under control conditions. Fifty T3 seeds of homozygous transgenic tobacco lines and wild-type were germinated on MS0 containing 100mg/L kanamycin. After a week, it was found that SAC1, SAC2, and SAC5 showed 82%, 87%, and 78% kanamycin resistant seedlings whereas all wild-type seedlings showed kanamycin sensitivity. Further, genomic DNA was isolated from leaf tissues of putative transgenic as well as non-transgenic (control) plants by CTAB method (Doyle and Doyle. 1987).

**3.2 Molecular testing of co-*AlaAT* transgenic tobacco lines**

Initial screening of putative transgenic plants was done by amplifying co-*AlaAT* gene specific fragment through PCR. Plasmid DNA was used as a positive (+ve) control while DNA from a non-transformed plant was used as the negative control. Amplification of gene specific 459 bp internal DNA fragment of the co-*AlaAT* gene indicated the successful transformation of this gene in five different transgenic lines (Figure 1, Lanes 1-5). However, data of only three lines (SAC1, SAC2, and SAC5) were presented in this manuscript. No amplification was observed in the non-transformed control (Wt: wild type).

Real-time PCR detection system (CFX96, Biorad USA) was used to quantify transgene expression in different transgenic tobacco lines. The acquired data was normalized with *18S* housekeeping reference gene. No expression of co-*AlaAT* gene was detected in wild-type (Wt) control, while the transgenic line SAC2 showed highest expression (4X higher expression than SAC1) followed by SAC1 and SAC5 (Figure 2).

**3.3 Co-*AlaAT* gene triggers morpho-physiological alterations in transgenic plants**

Selected transgenic tobacco lines were evaluated at 0, 75, 100 and 125 kg N/ha (N0, N75, N100 and N125) fertilizer doses through different molecular, morpho-physiological and biochemical means. Various morpho-physiological attributes like plant height, leaf area, stalk diameter, fresh mass, dry mass and seed weight were recorded and analyzed through two factor ANOVA of Microsoft Excel 2010 (Figure 3A-F). The values are mean of four replicates of tested transgenic lines at different nitrogen regimes. Plant heights of wild type control plants were significantly higher (at P<0.001) than transgenic plants under all nitrogen fertilizer doses at rapid vegetative growth (Figure 3A). However, upon reaching at maturity stage, plant height of wild type and transgenic plants differ non-significantly. The differences in the leaf area of fully developed (fifth) leaf of transgenic and control (non-transformed) tobacco plants (Figure 3B and Figure 5) were measured. At different N fertilizer regimes, transgenic plants showed significantly higher leaf area than wild-type control. The leaf area of control plants (Figure 3B) was significantly lower than that of all transgenic lines at N0 fertilizer application. SAC1 and SAC2 possessed highest leaf area at N0 and N75. However, SAC2 and SAC5 showed more leaf area at N100 and N125 fertilizer dose but increase in leaf area beyond N75 remained non-significant (Figure 3B).

Stalk diameter of SAC2 and SAC5 were significantly higher from SAC1 and wild-type at N0, N75, and N100 fertilizer dose while the stalk diameter of SAC1 was significantly greater than SAC2 and wild-type at N125 fertilizer level (Figure 3C). Fresh mass of SAC1 and SAC2 were significantly higher from wild-type and SAC5 at N0 fertilizer regime, however the fresh mass of all three transgenic lines was significantly higher than wild-type control plants at N75 fertilizer dose. At N100 and N125 fertilizer level, SAC1 and SAC2 retained significantly more fresh mass than SAC5 and wild-type control (Figure 3D).

Dry mass value of SAC2 was statistically higher from SAC1, SAC5 and wild-type control at N0 nitrogen fertilizer dose. Whereas dry mass values of SAC1 and SAC2 were statistically higher than SAC5 and wild-type control at N75. However, dry mass values of all transgenic lines remained significantly higher than wild type at N100 and N125 nitrogen regimes (Figure 3E). Seed weight of SAC2 was statistically higher than SAC1, SAC5 and wild type at N0 fertilizer dose, while seed weight of all transgenic lines were significantly higher from wild-type at N75, N100 and N125 fertilizer environment (Figure 3F).

**3.4 Co-*AlaAT* gene insertion elicit biochemical changes in transgenic plants**

Data of different biochemical parameters like total free amino acid, total nitrogen contents, crude protein and alanine aminotransferase activity was measured (Figure 4A-D). The experiment was repeated three times from year 2015-2017. The total free amino acids of SAC1, SAC2, and SAC5 were significantly higher than wild-type control at N0, N75, N100 and N125 fertilizer dose. However, level of total free amino acids was statistically similar in SAC1 and SAC5 but varied significantly with respect to SAC2 at all fertilizer regimes (Figure 4A). Total nitrogen content value at N0 fertilizer level was non-significant between wild-type and SAC1 but its values began to increase with the increase of nitrogen fertilizer application. The total nitrogen content of SAC1, SAC2, and SAC5 was significantly higher than wild-type at N75, N100 and N125 fertilizer level. However, SAC2 attained significantly higher nitrogen content than SAC1 and SAC5 at all nitrogen fertilizer applications. Total nitrogen content values reached to a maximum at N75 fertilizer regime in case of SAC2 but varied non-significantly at higher N fertilizer doses (Figure 4B).

SAC2 and SAC5 varied significantly from each other and had significantly higher crude proteins than SAC1 and wild-type at N0 fertilizer dose. Similarly, SAC1, SAC2, and SAC5 possessed significantly more crude protein than wild-type at N75, N100 and N125 fertilizer level (Figure 4C). Alanine aminotransferase activity of different transgenic lines was calculated at various nitrogen fertilizer exposure levels. All the transgenic lines possessed significantly higher alanine aminotransferase activity than wild-type control at all fertilizer levels. However, highest AlaAT activity was measured in SAC1, SAC2, and SAC5 lines at N75. Then its activity tend to be decreasing at N100 and N125 fertilizer doses. Lowest level of alanine aminotransferase activity was observed at N125 nitrogen regime in all transgenic and wild type lines, even then the level of AlaAT activity of all the transgenic lines was significantly higher than wild type plants at said fertilizer dose (Figure 4D).

1. **Discussion**

In an effort to improve nitrogen use efficiency (NUE) in crop plants, a codon optimized co-*AlaAT* gene was used in this study. Codon optimized *alanine aminotransferase* (co-*AlaAT*) gene was introduced and over expressed under *2XCaMV35S* promoter in tobacco (*Nicotiana tabacum* L.). Putative transgenic tobacco (T3) lines having optimum expression of co-*AlaAT* was used for evaluation under different N fertilizer regimes. The over expression of co-*AlaAT* gene resulted in significant increase in leaf area, stalk diameter, fresh and dry masses, root dry weight and seed weight as compared to non-transformed wild-type control under various N fertilizer doses. Wild type plants were using available N for increasing their heights during rapid vegetative growth stage at different N fertilizer applications. However, transgenic plants tend to accumulate and assimilate more N which was depicted in enhanced leaf area, stalk diameter and biomass produce. These results corroborate when a barley *AlaAT* gene was expressed under *OsAnt1* tissue-specific promoter in rice (Shrawat et al. 2008). A comparison of *2XCaMV35S*: co-*AlaAT* tobacco plants indicated that over expression of co-*AlaAT* gene showed association with the increase in leaf area, stalk diameter, fresh/dry mass produce (Figure 3A-F) at N0, N75, N100 and N125 nitrogen fertilizer applications. Contrary to that higher expression values of inserted gene some time does not result in the desired phenotype (Brauer et al. 2010). The differences in values for these attributes in transgenic plants were prominent under low level of nitrogen (N75) supply, whereas no or little differences were observed under high nitrogen fertilizer applications (Shrawat et al. 2008; Snyman et al. 2016). It is indicated that under optimal and overdose of N amendments (N100 and N125), the phenotypic and biochemical differences were minimum (Good et al. 2007). Lines SAC1, SAC2 and SAC5 exhibited highest amino acid, total N content, crude proteins and AlaAT activity (Figure 4A-D), which represented direct association with the moderate to the highest expression of co-*AlaAT* gene (Brauer et al. 2010) in these transgenic lines and finally resulted to improve nutrient use efficiency, phenotypic traits (McAllister and Good 2015) and biochemical attributes (Beatty et al. 2013). It has been demonstrated that AlaAT activity is involved in the production of either pyruvate or alanine for maintaining carbon-nitrogen balance (Miyashita et al. 2007) and control seed dormancy in the plant kingdom (Sato et al. 2016). Good et al. (2007) confirmed that total biomass, grain yield, and nitrogen contents in *Brassica napus* was enhanced by over expression of barley *AlaAT* cDNA under the control of root-specific promoter. In this pot experiment, transgenic tobacco plants sustained significantly higher biomass as compared to wild-type control and resulted in 25-30% decrease in nitrogen fertilizer requirement without causing any yield penalty. The results of expressing co-*AlaAT* in tobacco revealed many similar physiological, molecular and biochemical attributes as observed in transgenic brassica and rice (Good et al. 2007; Shrawat et al. 2008).

The increased fresh and dry biomass was evident from their high values for leaf area, stalk diameter, fresh/dry biomass, seed weight (Figure 3B-F), root dry mass and less internodal distance (data not shown here) values. It is implicated that reduced internodal distance in SAC2 and SAC5 as compared to wild-type might be due to co-*AlaAT* gene over expression for speedy translocation of essential nutrients from root to shoot. Root structure and size is an important parameter for ensuring efficient nutrient supply to various parts of shoot system. Transgenic tobacco plants retained densely arranged root system. SAC1, SAC2, and SAC5 possessed bushier, more lateral root proliferation, finer roots and root hairs as compared to wild-type (data not shown). Finer roots are reported to have more surface area to volume ratio as compared to thick root and plants nitrogen uptake is dependent on location, size and number of root hairs (Miller and Cramer 2004). It has been observed that availability of nitrate may interfere with the location and number of lateral root initiation sites (Malamy and Ryan 2001).

Transgenic tobacco revealed a significant increase in total free amino acid, nitrogen content, crude protein, and alanine aminotransferase activity. An increase in nitrogen flow was observed from Glutamine, Glutamate and Alanine when barley *AlaAT* gene expressed under the root-specific promoter in *Brassica napus* (Good et al., 2007). These amino acids are translocated to xylem sap immediately and trigger the plant growth. It is true for transgenic tobacco transformed with the co-*AlaAT* gene. Increase in alanine aminotransferase activity significantly increases the amount of total free amino acid, crude protein and total nitrogen content in transgenic lines. In shoot tissues an increase in the level of certain amino acids like Glutamate, Glutamine and Aspartic acid could be utilized as available nitrogen pool for the creation of nitrogen-containing compounds or other amino acids (Shrawat et al. 2008). Glutamine (Gln) is proposed to play a central role as a possible signaling molecule and in amino acid metabolism (Forde and Lea 2007). Co-*AlaAT* gene could play a vital role in starch synthesis under hypoxic or limited N condition (Yang et al. 2015).

SAC1, SAC2 and SAC5 lines confirmed significant improvement in N efficient phenotype when compared with wild-type control. Prominent N efficient phenotype was observed in transgenic lines SAC1 and SAC2 under limited N (N0 and N75) supply, indicating their efficiency to uptake available N while SAC2 and SAC5 were performing better at higher N doses ( N100 and N125) indicated their responsiveness to optimal or overdoses. These results are promising and support the application of transgenic approaches for improvement of nitrogen use efficiency in crop plants which could reduce the fertilizers input cost, increase the profitability in term of yield and decrease the environmental damage or probable risk to human health due to nitrates toxicity (Ahmed et al. 2017).

Optimal and efficient use of N fertilizer by plants is important to increase crop yield, to reduce fertilizers application and their hazardous impact on environment. The over expression of co-*AlaAT* gene enhances the N uptake, translocation and mobilization. It is therefore assumed that co-*AlaAT* gene could be transformed in major crop plants to enhance their N efficient phenotype.

1. **Author contributions**

MA conducted all experiments and wrote the first draft. MR and MYA helped in conducting physiological and biochemical testing of tobacco plants. ZM, SM, and NAS edited the paper and coordinated the research activities. All authors have read and approved the manuscript.

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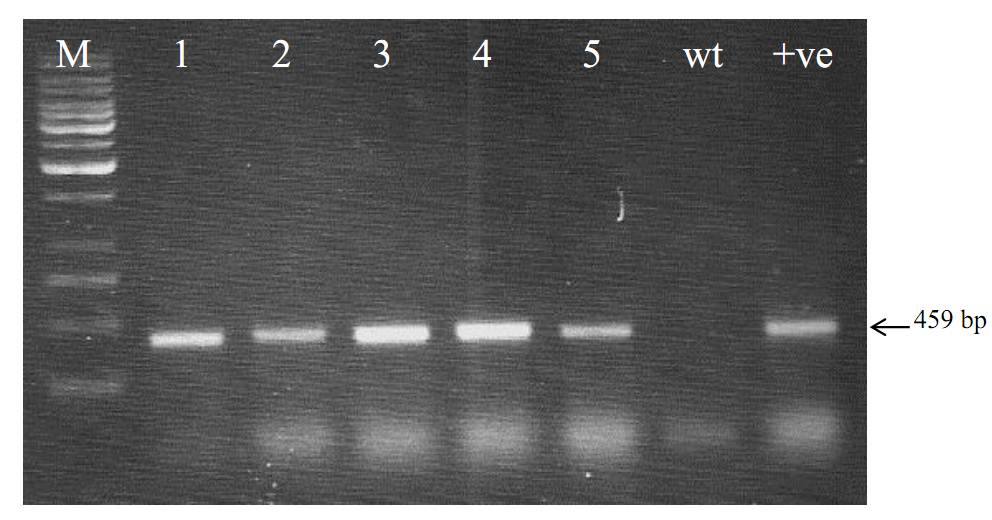
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Figure 1



Agarose gel electrophoresis showing the PCR amplification of 459 bp fragment of co-*AlaAT* gene from five independent transgenic events in tobacco. Lanes 1- 5 show gene amplification from SAC1, SAC2, SAC5, OAC2 and OAC3 transgenic plants (only first three lines described in this study), wt is a non-transformed negative control, +ve show gene amplification from plasmid control while M show 1 kb DNA ladder.

Figure 2

Relative quantification of co-*AlaAT* gene. Real Time PCR data using delta delta Ct (ddCt) method, representing average fold change of co-*AlaAT* transcripts level in different transgenic lines versus wild type (Wt) control.

Figure 3

Morpho-physiological assessment of; A. Plant height, B. Leaf area (fifth leaf from bottom), C. Stalk diameter, D. Fresh mass, E. Dry mass, F. Seed weight of wild type and putative transgenic co-*AlaAT* tobacco lines at N0, N75, N100, N125 kg/ha N fertilizer doses.

Figure 4

Biochemical evaluation of; A. Total free amino acids, B. Total nitrogen content, C. Crude proteins, D. AlaAT activity of wild type and putative transgenic co-*AlaAT* tobacco lines at N0, N75, N100, N125 kg/ha N fertilizer doses.

Table 1

|  |  |
| --- | --- |
| Primer Name | Primer Sequence (5’-3’) |
| *AlaAT*AF | TACTCCGCCTCTATCGCACT |
| *AlaAT* BR | CCCTCACTGGAGCTGAAAAG |
| *AlaAT* F | TTCTCTCCCCTTTGACGAGA |
| *AlaAT* R | TGATCGCATAACGCTAGCAC |
| *18S* F | TCTGCCCTATCAACTTTCGATGGTA |
| *18S* R | AATTTGCGCGCCTGCTGCCTTCCTT |

Table1. Primers used for PCR detection and real time quantification of co-*AlaAT* gene. *AlaAT*AF/*AlaAT* BR primer pair was used for PCR detection of co-*AlaAT* gene in putative transgenic tobacco plants while *AlaAT* F/*AlaAT* R and *18S* F/*18S* R primer pairs used for of co-*AlaAT* gene quantification in different transgenic tobacco lines.